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Physiological and kinetic characterization of a suspended cell anammox culture

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ABSTRACT

Anammox related technologies are currently widely applied for nitrogen removal from sewage sludge digester rejection water. Nevertheless, many aspects of the anammox process like the kinetic characteristics and the reaction stoichiometry are still subject of debate. Parameter values reported in literature are often hampered by mass transfer limitation or by the presence of a significant side population. In this study a membrane bioreactor (MBR) based method for growing a highly enriched anammox microbial community is described. The almost pure free-cells suspension of highly active anammox bacteria was used for detailed kinetic and stoichiometric analysis of the anammox process. The anammox culture enriched during this study had a biomass specific maximum growth rate of 0.21 d^{-1} which is higher than ever reported before in literature. Using an experimental methodology based on imposing dynamic process conditions combined with process modeling and parameter estimation, the intrinsic nitrite half saturation constant was identified to be as low as 35 μg-N L^{-1}. This was confirmed to be an accurate estimation in the pH range of 6.8–7.5.

1. Introduction

The anammox process is the anaerobic conversion of ammonium and nitrite to dinitrogen gas (van de Graaf et al., 1996) catalyzed by autotrophic deep-branching Planctomycetes (Strous et al., 1999). Anammox bacteria are usually considered slow-growing microorganisms with reported minimal doubling times of 4–15 days (Strous et al., 1998; van der Star et al., 2008). The high abundance of free anammox bacteria in marine environments (Schmid et al., 2007) show that there is no fundamental limitation for these bacteria to grow as free cells. Nevertheless, the enrichment cultures used to study these microorganisms typically consist of agglomerates or biofilms. Full-scale anammox reactor technology is heavily exploiting granular sludge based systems (van der Star et al., 2007; Wett, 2007). Granular sludge-based reactor design (Nicolella et al., 2000) leads to compact reactors, which combine a short hydraulic retention time (HRT) with a long and stable solids retention time (SRT). However, biofilm or granular sludge reactor systems are not the most suitable systems for investigating the intrinsic properties of the microorganisms (van der Star et al., 2008). The correct assessment of bio-kinetic parameters such as substrate

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affinities, maximum growth rate or maintenance need is hindered by the mass transfer limitations within the floc or granule (Harremoës, 1977; Chu et al., 2003). Transport limitations potentially lead to underestimation of the observed maximum specific growth rate (μ^{max}) (Characklis, 1990) and overestimation of the intrinsic affinity constant (K_S). Moreover the presence of an undefined side population and the occurrence of inactive or dormant cells at longer SRT complicate the derivation of intrinsic reaction kinetics of the organisms.

The full stoichiometry of the anammox metabolism currently used for bioprocess design and modeling purposes is based on Strous et al. (1998). In that study stable conditions (comparable to a steady state in a chemostat) were achieved in an SBR, enabling mass-balancing under defined conditions. However the reported degree of enrichment for anammox cells was only 74% and the carbon balance relied on a set of data (collected over a period of about 200 days) of dry weight measurements affected by high standard deviation (up to $\pm$50%). The electron balance of the measured conversion rates used to calculate the stoichiometric equation had an error of 15%. Until now this stoichiometry has not been checked independently. Kinetic parameters of interest such as the affinity constant for nitrite have not been properly reported in literature (Strous et al., 1998; van der Star et al., 2008). The parameter values currently known are probably adequate for design purposes but they may lead to systematic inaccuracy in competition studies (e.g. biofilm modeling studies). For the correct evaluation of such physiological and kinetic parameters the availability of a highly enriched (negligible amount of non-anammox bacteria present) suspended culture (no mass transfer limitation) of anammox bacteria is essential. The membrane bioreactor (MBR) enables cultivation of slow-growing microorganisms with full biomass retention but without a selection on settling ability and thereby floc or granular sludge formation. Moreover it is possible to operate an MBR at dilution rates close to the maximum growth rate of the concerned organisms, minimizing the effect of maintenance, decay and cryptic growth of a side population. Such a reactor system therefore is a valuable tool for identification of intrinsic stoichiometric and kinetic parameter values of slow growing microorganisms like anammox (van der Star et al., 2008). van der Star et al. (2008) was the first to report on the suspended growth of anammox bacteria in an MBR system. The suggested requirements for obtaining suspended cell growth were low levels of bivalent ions in the medium (i.e., calcium and/or magnesium) and the addition of yeast extract to the standard medium from van de Graaf et al. (1996). The study could not differentiate between the effects of these two factors since they were applied at the same time. In this study we used the MBR system from van der Star et al. (2008) to characterize in detail a suspension culture of anammox bacteria for the stoichiometric and kinetic characteristics. We used a method for kinetic characterization that is based on the combination of dynamic modeling and a continuous process exposed to dynamic conditions e.g. short time changes in nitrogen load. Provided that the experiment is started in a stirred tank reactor in steady state, the methodology described here allows for efficient kinetic characterization of a slow growing culture in a few days.

2. Materials and methods

2.1. *Inoculum*

The reactor was inoculated with granular sludge from the upper part of the lower compartment of the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, the Netherlands (van der Star et al., 2007). The reactor contains granular anammox sludge and treats reject water after partial nitritation in an SHARON reactor. The inoculum, was confirmed to consist of a "*Brocadia*" enrichment by *fluorescence in situ hybridization* (FISH), the sludge hybridized with AMX-820 and not with KST-157 oligonucleotide probes (Schmid et al., 2001). The reactor was inoculated with 1.8 L of settled granular biomass.

2.2. *Reactor operation*

The reactor was operated as reported by van der Star et al. (2008) with the following changes:

a) The liquid volume was 10 L (V_L) and the reactor was fed continuously with 6 L d^{-1} medium with different compositions, resulting in an HRT of 1.67 days.
b) The headspace volume was 5 L (V_H).
c) To maintain anoxic conditions and to provide buffering capacity, the reactor was sparged continuously at 50 mL min^{-1} with an industrially prepared mixture of Argon and CO_2 (95 and 5%, respectively). The gas entering the reactor was supplied from pressurized bottles with Brooks mass flow controller (MFC; Brooks Instrument, Hatfield, PA, USA).
d) The pressure inside the reactor was maintained constant at 12 hPa by connecting the outflow gas-tube to the bottom of a water-filled vessel to act as a water-lock. Since day 333 on a dithionite solution (200 mM) in which the gas (Argon/CO_2, 95/5%) was bubbling (through a "fine-bubbles" porous sparger) before entering the reactor was also implemented in the set-up.
e) During normal operation the pH was not controlled, but was always between 6.8 and 7.5. Since day 451 the addition of bicarbonate was discontinued and a phosphate pH-buffer (tot-P 15 mM) was added to the medium in order to set the pH at 7.0 (Table 1).
f) The temperature was controlled at 30 °C, and the stirring speed was 200 rpm.
g) The reactor was fed with a concentrated medium according to van der Star et al. (2007) with the changes reported in Table 1. The vitamin solution mentioned in Table 1 contained (mg L^{-1}): Folic acid (2.0), Riboflavin (5.0), Biotin (2.0), Thiamine (5.0), Nicotinic acid (5.0), Calcium Pantothenate (5.0), Vitamin B12 (0.1), p-Arninobenzoic acid (5.0), Thioctic acid (5.0), Monopotassium phosphate (900.0). Every time a new vessel of feeding-medium (20–50 L) was prepared, it was intensively sparged with nitrogen gas for 2–4 h before connecting it to the reactor.

The start-up period, from the day of inoculation (the designated experimental day 1) until the design volumetric nitrogen loading rate (1 g-N L^{-1} d^{-1}) was achieved, lasted one